Epothilones, a New Class of Microtubule-Stabilizing Agents with a Taxol-like Mechanism of Action

Research by D.M. Bollag, P.A. McQueney, J. Zhu, O. Hensens, L. Koupal, J. Liesch, M. Goetz, E. Lazarides, and C.M. Woods, Cancer Res. ***1995***, 55, 2325

Condensation and commentary by ***Suzanne B. Buck, Sam F. Victory, Richard H. Himes,*** *and* ***Gunda I. Georg,*** *University of Kansas*

Condensation of the Research

Purpose of the Study

To screen for different structural classes of compounds that would mimic the microtubule-stabilizing properties of paclitaxel

Background

Paclitaxel[1–4] is a novel chemotherapeutic agent approved in the United States for the treatment of ovarian cancer, breast cancer, and Kaposi's sarcoma. Additional clinical trials have shown promising results in patients with squamous cell cancer, urothelial cancer, esophageal cancer, and non-Hodgkin's lymphoma.[5]

The target of paclitaxel is the microtubule cytoskeleton. Microtubules (MTs) are eukaryotic cellular structures that are involved in the movement and positioning of chromosomes during mitosis, as well as the movement of vesicles and organelles. Paclitaxel promotes the self-assembly of tubulin into microtubules. It binds to the β-subunit of the tubulin heterodimer in microtubules in a 1:1 stoichiometry and stabilizes the MTs. The effects of paclitaxel on cells are dependent on the concentration of the taxane. At high concentrations, paclitaxel upsets the microtubule–tubulin balance by causing the formation of an excess of microtubules that tend to bundle together.[6] The microtubules in the cell are stabilized, preventing the formation of the mitotic apparatus. Low concentrations of paclitaxel block mitosis by binding to the microtubules in the mitotic apparatus and inhibiting their dynamics.[7] Cells treated with paclitaxel eventually die by the process of apoptosis.[8]

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The development of paclitaxel as an anticancer drug was difficult because isolation of paclitaxel from the bark of the Pacific yew yields only 0.014% paclitaxel.[9] However, semisynthesis of paclitaxel, starting from 10-deacetylbaccatin III (10-DAB), obtained from a regenerable source, the needles of *Taxus baccata*, yields enough paclitaxel to alleviate the supply problem.

Paclitaxel has a number of clinical side effects such as neutropenia, peripheral neuropathy, alopecia, and hypersensitivity reactions.[10] The hypersensitivity reactions are apparently related to the current formulation of paclitaxel with the surfactant Cremophor EL®, which is needed to increase the aqueous solubility of paclitaxel.[10] Paclitaxel's clinical efficacy may be reduced in some patients because it is a substrate for P-glycoprotein, which is present in multidrug resistant (MDR) cancer cells. P-glycoprotein is an energy-dependent transmembrane protein that pumps many lipophilic natural product drugs from MDR cells.[11]

After paclitaxel's discovery, it was thought that other natural products must exist that have a mechanism of action similar to that of paclitaxel. Bollag et al. at Merck therefore designed a screen that detects microtubule-nucleating activity at low concentrations and that can be used for high-throughput screening programs (300 compounds per day can be screened by one worker). The test compounds were incubated with tubulin, and the protein polymer that formed due to the presence of the active test compound was collected. Protein concentration was measured by absorbance spectroscopy after staining. This screen tests compounds directly for their ability to bind and to induce microtubule formation. The assay is very sensitive, measuring microtubule stabilization activity as low as 100 nM concentration. With this new screen, Bollag et al. set out to identify new natural products that mimic paclitaxel's ability to induce the formation of MTs from tubulin and to hyperstabilize them.

What Researchers Accomplished

The researchers:

- Developed a high-throughput screening method to identify tubulin-stabilizing agents. The sensitivity of the assay is about 10 times greater than traditional methods.
- Screened over 7,000 compounds from plant, marine, insect, and microbial extracts in less than 6 months, using the novel, high-throughput screen they developed, and identified activity in the fermentation extracts of *Sorangium cellulosum* strain SMP 44.
- Determined the structures of epothilones A and B using spectroscopic techniques.
- Demonstrated that the epothilones induce tubulin polymerization at concentrations similar to paclitaxel in turbidity and sedimentation assays and that depolymerization of microtubules treated with either epothilone did not occur at 4°C or in the presence of calcium.
- Demonstrated that epothilones A and B are competitive inhibitors of paclitaxel.

- Determined that the mechanism of action of the epothilones is similar to paclitaxel, blocking the G2 to M transition in the cell cycle.
- Observed levels of cytotoxicity induced by epothilones A and B at concentrations comparable to paclitaxel in several cell lines.
- Showed that epothilones A and B retained significant activity against the MDR cell line KBV-1 that has elevated levels of P-glycoprotein as compared to paclitaxel, which had a 20,000-fold decrease in its activity against the MDR cell line.

Researchers' Approach

Bollag et al. designed a high-throughput screen for large-scale testing of plant, marine, insect, and microbial extracts. The assay is highly sensitive and identifies microtubule nucleating activity in concentrations as low as 100 nM, using a filtration-colorimetric assay to detect tubulin polymerization induced by test compounds.

An extract of *S. cellulosum* strain SMP 44 was screened and found to possess microtubule-nucleating activity. Fractionation of this extract resulted in the isolation of two myxobacterial metabolites, epothilone A and its C-12 methyl homolog, epothilone B (Fig. 1). Prior to this study, Höfle and co-workers[12] had isolated epothilones A and B from an extract of *S. cellulosum* strain So ce90 (present in soil samples obtained from the banks of the Zambesi River in the Republic of South Africa) in their screening program for antifungal metabolites.

The epothilones were compared to paclitaxel in a number of biochemical assays and were found to be almost identical in their effects on tubulin polymerization and cytotoxic properties. However, they were significantly more potent against several paclitaxel-resistant cancer cell lines.

The characteristics of the epothilone-induced polymerization were studied using turbidity and sedimentation assays, demonstrating that the induction of polymerization occurred at concentrations similar to those of paclitaxel. Tubulin polymerization was verified by electron microscopy.

Like paclitaxel, the epothilones stabilize microtubules against cold- or calcium-induced depolymerization. In competition assays, epothilones

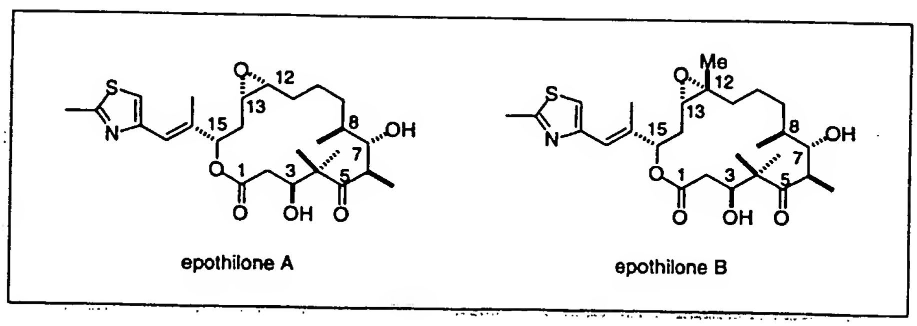

Figure 1. The structures of epothilone A and epothilone B as determined by X-ray crystallography.[13]

Table 1. Results of *in vitro* studies of epothilones' cytotoxicity (EC_{50}) compared to paclitaxel[14]

Cell line tested	Paclitaxel	Epothilone A	Epothilone B
KB3-1	1.2 nM	13 nM	15 nM
KBV-1	23,000 nM	160 nM	58 nM

A and B prevented the binding of [^{3}H]paclitaxel, demonstrating that the epothilones are competitive inhibitors of paclitaxel and, thus, compete with paclitaxel for the same or an overlapping binding site.

A study of the time course of mitotic arrest and cytotoxicity demonstrated that the epothilones and paclitaxel have identical kinetics of G2-M block and identical lag and kinetics of cytotoxicity. It was also found that the epothilones, like paclitaxel, cause cell death by apoptosis. The epothilones induced random microtubule bundling in the cytoplasm similar to what is observed for paclitaxel.

Epothilones A and B were tested on the MDR human carcinoma cell line KBV-1 (Table 1).[14] It is known that cells exhibiting MDR contain the P-glycoprotein that decreases drug concentration within the cell. The KBV-1 cell line contains elevated levels of the P-glycoprotein. The human carcinoma cell lines containing the P-glycoprotein were treated with epothilone A, epothilone B, and paclitaxel. Paclitaxel exhibited an approximate 20,000-fold drop in potency against the MDR KBV-1 cell line, as compared to the cytotoxicity against KB3-1, the parental line for KBV-1 that does not have elevated levels of the P-glycoprotein. Epothilones A and B exhibited only a 4- to 12-fold drop in potency against the KBV-1 cell line as compared to the cytotoxicity induced in the KB3-1 cell line.

This assay demonstrated one of the major advantages of epothilones A and B over paclitaxel; epothilones A and B are less effective substrates for the P-glycoprotein and are, therefore, not transported out of the MDR cells as quickly as paclitaxel.[15]

Commentary on the Research

The development of a novel, high-throughput screen for microtubule-nucleating activity is important because it can be used to quickly identify microtubule stabilization induced by low concentrations of test compounds. The screen increases the number of samples that can be identified as compounds that induce hyperstabilization in a manner similar to paclitaxel.

There had been no reports of other compounds that bind to tubulin and induce hyperstabilization similar to paclitaxel until the discovery of epothilones A and B as the first nontaxoid compounds with that mechanism of action. More recently, discodermolides[16] and the eleutherobins[17] have also been found to mimic paclitaxel's mechanism of action on microtubules.

The interesting bioactivity of the epothilones has stimulated further research. Kowalski et al.[15] tested the human colon carcinoma cell line SW620AD-300, which also expresses high levels of P-glycoprotein. The epothilones had very little loss of activity against the MDR cells as compared to paclitaxel's 1,000-fold decrease in activity. Epothilone B was more potent against the MDR cell line in this study. This group also disclosed that the epothilones are active against the paclitaxel-resistant ovarian carcinoma cell line 1A9(PTX22), which expresses a modified β-tubulin.[15] Thus, these epothilones have the potential of overcoming two different mechanisms of resistance to paclitaxel.

The results of the tubulin-binding studies would suggest that the binding sites for paclitaxel and epothilone are identical. However, the fact that the epothilones are effective cytotoxic agents in cells that are resistant to paclitaxel because of the expression of an altered β-tubulin indicates that the binding sites may be overlapping rather than identical. Photoaffinity labeling studies have shown that paclitaxel has its major binding site on β-tubulin.[18,19] These results were confirmed recently by the publication of the structure of the αβ-tubulin dimer by electron crystallography (3.7-Å density map), in the presence of paclitaxel, that showed binding of paclitaxel to the β-subunit.[20]

A recent publication by Mühlradt et al.[21] demonstrated that the epothilones do not possess the endotoxin-like activity of paclitaxel described previously. Because the endotoxin-like properties of paclitaxel are most likely the cause of unwanted clinical side effects such as myalgia/anthralgia, the epothilones have the potential to be clinically superior to paclitaxel.

The identification of epothilones A and B as paclitaxel-like compounds led to an extensive NMR study using phase sensitive DFQ COSY and NOESY and distance constrained molecular-modeling techniques to define the relative stereochemistry of the epothilones.[22] The X-ray crystal structure was published by Höfle et al.,[13] and the absolute stereochemistry of the compounds was revealed.

Subsequently, a significant number of publications dealing with the synthesis of the epothilones and related analogs have occurred, which are discussed in the article by Appendino and Casiraghi in this issue.

The discovery of epothilones has also led to studies comparing the very different structures of paclitaxel and epothilone to determine a common pharmacophore. The epothilones have only one macrocycle, whereas the taxanes are comprised of four separate rings. The taxanes are approximately twice the molecular weight of the epothilones, and contain twice as many oxygen molecules. The taxanes and epothilones do have similarities; both possess aromatic side chains, a keto group, a gem-dimethyl group, and an ether ring. These similarities have been examined using molecular-modeling techniques. Winkler and Axelsen[23] were able to superimpose 13 of the 15 ring atoms of epothilone and a majority of the side-chain atoms onto corresponding atoms in paclitaxel.

The epothilones are a class of compounds that may prove to be more effective chemotherapeutic drugs than paclitaxel with less, unwanted,

clinical side effects. The epothilones are readily obtained from fermentation, eliminating any supply problem. An initial in vivo evaluation demonstrated that epothilone B has a significant advantage over paclitaxel in the reduction of tumors in SCID mice bearing drug-resistant human CCRF-CEM/VBL xenografts.[24] Epothilones are approximately 30 times more water soluble than paclitaxel, and their structures can be more easily altered to increase pharmacological properties because the epothilone macrolide structure is more amenable to chemical modifications than the complex taxane structure. With the discovery of epothilones A and B, it is hoped that more questions will be answered about paclitaxel and epothilone's mechanism of interaction with microtubules, providing a starting point for further drug development.

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